MOLECULAR WEIGHT DETERMINATION OF COMPONENT I OF THE BLOOD SERUM OF DIETHYLSTILBESTROL-TREATED COCKERELS

by

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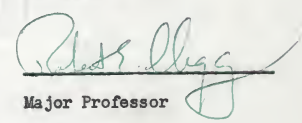

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TABLE OF CONTENTS

INTRODUCTION 1

LITERATURE REVIEW 2

MATERIALS AND METHODS 6

Preparation of Serum 6

Preparation of Bottom Fraction 6

Preparation of Component 1 7

Vertical Starch Gel Electrophoresis 9

Thin-layer Chromatography 11

Extraction of Lipid 11

Preparation of the Chromatographic Plates 11

Developing of Chromatogram, two-dimensional 12

Detection of Components 12

Gel Filtration on Sephadex Columns 12

Reagents 12

Packing of the Column 13

Examination of the Homogeneity of the Column 15

Determination of Flow Rate 15

Application of Sample to the Column 15

Examination and Measurement of the Elution Volumes 16

EXPERIMENTAL AND RESULTS 18

Separation by Ultracentrifugation 18

Purification by Density-gradient Centrifugation 18

Characterization by Vertical Starch Gel Electrophoresis 21

Application of Thin-layer Chromatography 24

Determination of Void Volume 24

Elution volumes of Proteins of Known Molecular Weights 27

Calibration of Sephadex Columns for Molecular Weight Determination 32

Determination of Molecular Weight of Component 1 32

DISCUSSION 41

SUMMARY 45

ACKNOWLEDGMENT 46

BIBLIOGRAPHY 47

INTRODUCTION

Various investigators noted that the sera of laying hens and diethylstilbestrol-treated cockerels contained two additional electrophoretic components which were not present in the sera of non-laying hens and normal cockerels. The appearance of these components was assumed to be related to egg formation and was paralleled by an increase in the calcium, phosphorus and total protein content of the sera. One of these components has been designated as component 1 (prealbumin) because it appeared in front of the albumin during free electrophoresis. The other component has been designated as component 6 and was found in the same area as beta-globulin in the electrophoretic pattern. After ultracentrifugation, component 1 has been found in the reddish, semi-solid precipitate at the bottom portion of the centrifuge tube, and component 6 has been found in the yellowish, waxy substance in the top portion of the tube.

By employing a combination of ultracentrifugation and density-gradient centrifugation, a small amount of component 1 has been fractionated. This investigation was initiated to prepare larger amounts of this component and to determine the molecular weight.

LITERATURE REVIEW

Numerous attempts were made to investigate the electrophoretic patterns of the blood sera of laying hens, non-laying hens, cockerels and hormone-treated roosters. Deutsch and Goodloe (12) studied chicken plasma and obtained a different electrophoretic pattern for females and males. They found that in the female electrophoretic pattern, there was an additional component moving in front of the albumin and that the size of beta-globulin fraction was increased. This characteristic electrophoretic pattern appeared only with laying hens and increased significantly with age. Moore (30), and Kibrick and Blonstein (24) confirmed these results and found that the content of total protein and beta-globulin fraction in the serum increased with age. These results were also obtained by Brandt, Clegg and Andrews (5) and they further indicated that the total protein, alpha-globulin fraction, and beta-globulin fraction increased with age, while the albumin, and the gamma-globulin fraction remained relatively the same. Moreover, Brandt et al (5) observed that in the sera of laying hens, there was a fast moving fraction not evident in the sera of non-laying hens and cockerels. The fast moving component was thought to be associated with egg formation because it appeared at the onset of egg production. In a study employing pigeons, Sendroy, Mackenzie and Collison (40) showed the appearance of a fast moving component and an increase in the beta-globulin area in the sera of pigeons during egg production.

The serum of a cockerel treated with feminizing hormone produced an electrophoretic pattern similar to that of the laying hen (31) (8). Clegg et al (8) investigated the effect of estrogen-like substances and noticed that the injection of diethylstilbestrol to cockerels produced an electrophoretic pattern similar to that of laying hens. The appearance of the fast moving fraction designated as component 1 and the other component in

the beta-globulin area of the pattern (component 6) indicated that the injection of diethylstilbestrol would cause changes associated with egg production in the serum protein of roosters.

Ether extraction had an effect on the chicken serum. McFarlane (28) found that some electrophoretic components were reduced or disappeared completely with ether extraction. Moore (30) compared the electrophoretic patterns of chicken sera before and after ether extraction and observed that much of the lipid was removed from the sera of laying hens or hormone-treated birds. Ericson (13) used ether extraction to study the properties of component 1 and component 6. He found that the electrophoretic pattern of diethylstilbestrol-treated cockerels changed in the same manner as the pattern of the laying hens which showed a disappearance of component 1 and a decrease in the size of beta-globulin area. Therefore component 1 and component 6 were thought to be lipoproteins. Based on the interaction between lipoprotein and macromolecular sulfated polysaccharides, lipoprotein could be removed by centrifugation (33) (2). Homma (21) removed the lipoprotein of laying hen serum and diethylstilbestrol-treated non-laying hen serum by the addition of the sodium salt of dextran sulfate. However, with sodium dextran sulfate treatment, Wu (50) found that component 1 did not precipitate with anionic polysaccharide molecules, and therefore according to this criteria component 1 was not a lipoprotein.

In a series of studies with moving boundary electrophoresis and column electrophoresis, Chen (7) found that component 1 was mainly concentrated in the "Bottom Fraction" after ultracentrifugation of the blood sera of laying hens and diethylstilbestrol-treated cockerels. Chen (7) further prepared a small amount of electrophoretically homogeneous component 1 employing moving boundary electrophoresis, salt fractionation and column electrophoresis.

Wu (50) attempted to obtain a larger amount of component 1 which was electrophoretically and chromatographically homogeneous, by a combination of methods of electrophoresis, density-gradient centrifugation and DEAE-SF anion exchange chromatography.

Syne and Tieslius (46) first observed the molecular sieve effect in gels with fractionation of hydrolytic products of amylose in agar-agar jelly. Mould and Synge (32) found that the escape time of synthetic amyloses from collodion membranes was proportional to molecular weight. It was also reported by Craig, King and Stracher (9) that the escape time of a number of proteins through cellophane membrane was a function of molecular weight of the protein. Lathe and Ruthvan (25), by determining the elution volumes of a number of proteins from water-swollen starch columns, suggested that the dimensions of proteins might be determined by this technique. However, it was difficult to evaluate their suggestions due to the uncertainty in size and shape of the macromolecules in solution (38). Using a granulated latex rubber column, Brewer (6) observed a linear relationship between corrected elution volumes and the molecular weight of low molecular weight polymers. For a homogeneous series of macromolecules, the size and molecular weight were closely related, as indicated by a relation between molecular weight and gel filtration behavior of dextrans (18). Granath and Flodin (18) found a linear correlation existing between the log of molecular weight and the ratio of elution volumes to total bed volume. Andrews (1) confirmed their evidence with agar-gel columns and further showed that gel filtration could be used as a comparative method for estimation of protein molecular weight. Andrews (1), and Steers and Ackers (45) suggested that a determination of molecular weight of a protein could be done more readily by the use of agar gel filtration which would permit a much better separation of proteins. Flodin (14), and

Flodin and Porath (15) definitely separated solutes on cross-linked dextrans according to molecular weight.

Whitaker (48) studied proteins and enzymes with known molecular weights and established an excellent linear correlation between the log of molecular weight of proteins employed and the ratio of elution volume, V, to the void volume, Vo, of a Sephadex column, G-100. The ratio of elution volume to void volume, V/Vo, was independent of column size though both V and Vo varied. The ratio V/Vo was also found to be constant with different sizes and concentrations of proteins. Ion exchange adsorption had a minor effect on the ratio V/Vo, but at an ionic strength of 0.494 this effect was negligible. It was found to be temperature dependent for some proteins such as cytochrome C and alpha-chymotrypsin.

In the following experiment, the gel filtration technique was employed to determine the molecular weight of component 1 of the blood serum of diethylstilbestrol-treated cockerels.

MATERIAL AND METHODS

Preparation of Serum

Sera used in this investigation were obtained from the blood of White Rock cockerels fed a commercial chick ration. The birds were from three months to one year old. The cockerels were injected, subcutaneously, with 7.5 mg per day of diethylstilbestrol in 0.5 ml of propylene glycol for a period of eight days. At the end of this period they were sacrificed. The blood was collected in 50 ml centrifuge tubes and allowed to clot at 38° C. for 60 minutes. The blood was centrifuged at 5,000 r.p.m. for 30 minutes at 0° C. The serum was decanted into a graduated cylinder, covered and stored at 5° C. overnight.

Preparation of the Bottom Fraction

Ultracentrifugation was used in the initial step of separating the components of the whole serum. Polyallomer or lusteroid cellulose tubes, 5/8"x 3", were filled with the serum to about 1/8" from the top. The tubes were tightly capped with aluminum caps, placed in a pre-cooled centrifuge head (No. 40 rotor), and centrifuged at 38,000 r.p.m. for 16 hours at 0° C. The instrument used was a Spinco Preparative Ultracentrifuge, Model L.

After centrifugation three distinct fractions were observed. These fractions were designated as "Top Fraction", "Middle Fraction", and "Bottom Fraction" respectively. Chen (7) found that the "Bottom Fraction" contained a high concentration of component 1. Chen (7) and Wu (50) prepared a purified, electrophoretically homogeneous, component 1 from the "Bottom Fraction". Misra (29) and Malik (26) prepared a homogeneous component 6

from the "Top Fraction".

After removing the "Top Fraction", a solid, waxy substance, with a spatula and decanting the viscous liquid of the "Middle Fraction", the "Bottom Fraction", a semi-solid precipitate, remained in the bottom of the centrifuge tube. The "Bottom Fraction" was dissolved in a minimum amount of borate-chloride buffer, pH 8.6. The composition of the buffer was as follows: 0.05 M potassium chloride, 0.05 M borric acid, and 0.012 M sodium hydroxide. The pH of the buffer was adjusted to pH 8.6 with sodium hydroxide and / or hydrochloric acid.

Brandt, Clegg and Andrews (5) found that borate-chloride buffer was better for the electrophoretic analysis of component 1 from blood serum than any other buffer commonly used in the electrophoretic studies of serum protein.

Preparation of Component 1

Component 1 was concentrated in the "Bottom Fraction" after ultracentrifugation. It was further separated from other components by density-gradient centrifugation. Density-gradient centrifugation has been used widely for the separation of viruses, cell particules, serum and lipoproteins. Brakke (4) first employed density-gradient centrifugation to separate potato yellow-dwarf viruses.

Density-gradient centrifugation separates the components of mixtures of several discrete fractions in a sample by their differences in density. A steep gradient column of some solvent and solute in which the particles are soluble is required. The particles to be studied are layered on the top of the gradient column and are prevented from sedimenting in the liquid by the steep positive density gradient. When centrifugal force is applied,

the particles will form zones in the fluid column. These zones of particles will be separated from one another depending on density. After centrifugation, each zone can be removed separately for further analysis.

An ideal gradient-forming material is non-toxic and chemically inert to the particles to be studied. The gradient-forming material should also be readily soluble in water and salt solution, of low viscosity and of high density and molecular weight. Low viscosity will permit a rapid sedimentation and promote ease of pipetting during the gradient formation, as well as ease of fractionation by drop-counting. A high density is necessary for the formation of a steep gradient, and a high molecular weight is required to minimize osmotic pressure gradient in concentrated solutions. If the particles are to be analyzed later for total protein content, nitrogen should be absent in the gradient material (43).

In this experiment, sucrose solution was used as the gradient-forming material. Although sucrose has a high viscosity, it has been widely used for the formation of gradient. Sucrose solutions of 10%, 20%, 30%, 40%, and 50% in the borate-chloride buffer solution, pH 8.6, (page 7) were employed. By means of a pipette, 5 ml aliquots of each of the sucrose solutions were introduced into lusteroid centrifuge tubes of 1" x 3", in the order of their increasing density. It is necessary to introduce the sucrose solution slowly and steadily in order to obtain a distinct boundary between the consecutive different concentrations. After the gradient was formed, 5 ml of the sample (the "Bottom Fraction" in the borate-chloride buffer solution, pH 8.6, after ultracentrifugation) were added as droplets onto the top of the gradient column. The centrifuge tubes were covered tightly with aluminum caps and fixed onto a pre-refrigerated centrifuge head, No. 25-D roter, a swinging bucket rotor. The centrifugation was carried out in a Spinco

Preparative Ultracentrifuge at 16,000 r.p.m. for 16 hours at 0° C.

After centrifugation, several fractions visible as bands in the sucrose solution and a yellowish precipitate were obtained. Previous work indicated that the precipitate contained component 1 and therefore the yellowish precipitate from each centrifuge tube was dissolved in a minimum amount of borate-citrate buffer, pH 6.0 (page 13) and combined. This precipitate was designated as component 1 by Chen (7) after a series of investigations with moving boundary and column electrophoresis.

A portion of component 1 was analyzed by means of starch gel electrophoresis and thin-layer chromatography. The remainder of component 1 was dialyzed against borate-citrate buffer, pH 6.0, and employed in the molecular weight determination by gel filtration on Sephadex column.

Vertical Starch Gel Electrophoresis

The apparatus for the vertical starch gel electrophoresis was manufactured by Buchler Instruments, Inc. Fort Lee, New Jersey. The technique employed in this experiment followed the method of Boyer (3).

Hydrolyzed starch[1] (45.45 gm) was suspended in 450 ml of gel buffer (page 10) in a thick-walled suction flask. While stirring and swirling constantly to prevent lumpiness, the suspended starch was heated to 75° C. After the flask was degassed for 30 seconds, the starch was poured into a lucite tray with both end plates and comb assembled beforehand. It was immediately covered with a prewarmed glass plate and allowed to stand undisturbed for 3 hours at room temperature. Both the lucite tray and the glass cover plate were lightly oiled for better removal of starch gel.

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1. Lot 208-1 from Connaught Medical Research Labs., U. of Toronto, Toronto, Canada.

A maximum of 10 samples could be applied at one time into the slots provided in the mold. The comb was removed and approximately 0.04-0.045 ml of sample was applied by means of a micropipette. After the application of the sample a solidifying-vaseline-melt was quickly layered on the sample slots to prevent evaporation.

Both the upper and lower electrode chambers were filled with 400 ml of electrode chamber buffer (page 10, see below). Several strips of filter papers, Whatman No. 3, were placed in the lower chamber to provide for current continuity. The lucite mold with both end plates and the comb removed was erected vertically against the lucite stand with samples on the upper part. Flannelettes serving as wicks were placed from the upper electrode chamber to the upper end of starch gel. Electrophoresis was conducted at a current of 15 ma, for 16 hours, at room temperature.

The starch gel was sliced into three parts horizontally by means of the gel slicing device, and the middle portion used for the staining procedure. The thickness of the middle layer of starch gel ranged from 2 to 3 mm.

The gel was stained with amido black dye for 1 hour and then washed with amido black wash solution until the starch was clear. The starch gel was soaked in glycerine solution for at least a week in order to make the unstained portion of the starch gel transparent before taking pictures.

Buffers used for the starch gel electrophoresis were (3):

1. Gel Buffer: the composition was 0.025 M boric acid and 0.01 M sodium hydroxide. The pH was 8.6-8.7.
2. Wick and Electrode Chamber Buffer: this buffer was prepared by dissolving 18.5 gm of boric acid and 2.5 gm of sodium hydroxide pellets in 1 liter of distilled water (The Smithies' original system and the

Poulik' system). pH = 8.0-8.2

Amido Black 10B obtained from Hartman-Leddon Co. in Philadelphia, Pennsylvania, was used in staining the starch gel electrophoresis. The staining solution was prepared by saturating Amido Black 10B in a solution consisting of 95% ethanol, glacial acetic acid and water (50 : 50 : 10 v/v) (42). The wash solution for Amido Black stain consisted of 95% ethanol, water, glacial acetic acid in the ratio of 50 : 50 : 10 v/v (42).

Thin-layer Chromatography

Extraction of Lipid. A modification of a method of Selvey (39) was used. One ml of component 1 and 17 ml of a mixture of chloroform and methanol (1 : 1) were mixed in a 50 ml flask and allowed to stand for 10 minutes, then placed in a hot water bath until boiling occurred. After cooling, the mixture was filtered into a centrifuge tube and washed with 8 ml chloroform. Four ml of water were added with shaking. Centrifugation was carried out at 15,000 r.p.m. for 10 minutes. After centrifugation, the water layer was aspirated, and the volume evaporated to dryness under nitrogen pressure in a warm bath. The residue was dissolved in a minimum amount (a few drops) of chloroform. This sample was used for the two-dimensional thin-layer chromatography to be described subsequently.

Preparation of the Chromatographic Plates. Silica gel G[1] (30 gm) was first suspended in 40 ml of distilled water, then an additional 20 ml of distilled water was added, and mixed well. A thin slurry of the silica gel was poured by means of a rectangular spreader and a trough onto the surfaces of five square glass plates (20 cm x 20 cm) lined up on an aligning tray. The

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1. No. 8076, from Research Specialties Co., California.

films were 250 microns thick. The chromatographic plates were placed on racks and heated to 105°-110° C. for 60 minutes to reactivate the adsorbent. The sample to be analyzed (the lipid extracted from component 1) was placed on the film by means of a micropipette.

Developing of Chromatogram, two-dimensional. Two chromatographic plates were placed in a support rack and put into a chromatographic tank containing 200 ml of the first developing solvent of chloroform : methanol : water in the ratio of 65 : 25 : 4 (v/v). After the chromatogram was developed to a predetermined point (15-16 cm), which took approximately 75 minutes, the plates were removed from the tank and allowed to evaporate to dryness, at room temperature under a hood.

The chromatographic plates were turned 90° and placed into a second chromatographic tank containing 200 ml of the second developing solvent of heptane : ether : acetic acid in the ratio of 80 : 20 : 6 (v/v). The plates were allowed to develop for about 45 minutes, then removed and evaporated to dryness as before.

Detection. Iodine vapor was used to detect the components. The chromatograms were immersed in an enclosed tank containing a few crystals of iodine. The iodine vapor was absorbed by the silica and the location of the components revealed by differential absorption.

Gel Filtration on Sephadex Columns

The proteins used for calibrating the standard curves on Sephadex columns were bovine gamma-globulins (fraction II), pepsin (2X crystalline, from dilute alcohol), trypsin (2X crystalline, salt free), beef pancreas alpha-chymotrypsinogen (6X crystalline, salt free), bovine pancreas ribonuclease (5X crystalline, salt free and without protease), and horse heart

cytochrome C (amorphous powder, containing 0.35% iron). All were obtained from Mann Research Laboratories, Inc., New York 6, New York.

Cross-linked dextrans for the columns were Sephadex G-100 (40-120 microns), Lot Number TO 2946, Sephadex G-200 (40-120 microns) Lot Number TO 3380, Blue Dextran 2000. All were supplied by Pharmacia Fine Chemicals, Inc., New Market, New Jersey.

Borate-citrate buffer, pH 6.0, was used as the eluting agent in the Sephadex gel filtration columns. The borate-citrate buffer was prepared by dissolving sodium tetraborate (19.07 gm) and sodium citrate (29.41 gm) in 1 liter of water. The pH of this buffer ranges from pH 6.0 to pH 9.6. The solution was adjusted to pH 6.0 by the addition of solid citric acid and/or sodium hydroxide (42).

Packing of the Column. Two columns (1.8 cm x 190 cm and 2.5 cm x 100 cm) were used in this investigation. The column, 1.8 cm x 190 cm, was a local product and the other column, 2.5 cm x 100 cm, was obtained from Pharmacia Fine Chemicals Inc., New Market, New Jersey. The packing of these two columns were similar except where indicated.

Sephadex-gel-filtration media G-100 particle size (33 gm) or G-200 particle size (17 gm) was suspended in borate-citrate buffer solution, pH 6.0, and allowed to swell for 48 hours in the case of G-100 or 72 hours in the case of G-200, with frequent stirring.

The column was mounted vertically and supported firmly. The lower outlet was closed with a screw clamp and the column was partially filled with borate-citrate buffer, pH 6.0. Any air bubbles in the bottom part were removed by opening the outlet and allowing the eluant to flow through the column. A thin slurry of de-aerated gel particles in buffer solution was poured into the column. The excess buffer was allowed to percolate

through the gel bed. The Sephadex gel was added continuously until a desired bed height of about 93 cm in the case of the short column, or 190 cm in the case of the long column was attained. A horizontal gel surface was made by stirring the upper portion of the bed and then allowing the gel to settle under gravity or by tapping on the column lightly. The column was equilibrated by letting about two column volumes of eluant to pass through the column. An eluant reservoir of borate-citrate buffer, pH 6.0, was connected to the top of the column and the flow rate was maintained at a rate of approximately 18 ml/hr. By the time the gel bed settled down the flow rate would decrease slightly. Both the upper inlet and lower outlet spouts were attached to a polyethylene tube.

With the 2.5 cm x 100 cm column, a sample applicator of Perspex was inserted into the column tube and was allowed to settle to the top of gel bed. The nylon net on the sample applicator allowed an even distribution of the sample applied over the gel surface and also served as stabilizer of the gel surface. The sample applicator was not necessary with the 1.8 cm x 190 cm column.

The flow rate was affected by the porosity of the Sephadex gel. In general, the flow rate increases with particle size and decreasing porosity of the gel. It is almost inversely proportional to the bed height. One way of adjusting flow rate is by the application of positive hydrostatic pressure to the top of the column. The hydrostatic pressure could be adjusted by the position of the eluant reservoir. But for a porous gel such as G-100 and G-200, a better result was obtained at low pressure. The total hydrostatic pressure for these gels was recommended to be not higher than 10-20 cm (41). This pressure was attained by lifting the outlet tube to a level of 10-20 cm below the level of the eluant in the solvent reservoir.

Too high a flow rate caused zone spreading and gave a poor resolution.

Examination of the Homogeneity of the Column. The homogeneity of the column was checked by watching the passage through the column of a blue Sephadex (Blue Dextran 2000), or a colored protein such as cytochrome C. The irregularity of the bed was readily detected by skewness of colored bands, or poor definition of peak maxima in elution diagram. Any irregularity was corrected by replacing the top 0.5-1 cm of gel with fresh gel and then stirring up the top 2-3 cm and allowing the gel particles to settle again with the eluant.

The well packed and homogeneous Sephadex gel column was kept at a constant temperature of 5° C. and was eluted continuously with borate-citrate buffer solution, pH 6.0, even when not in use.

Determination of Flow Rate. An accurate measure of the elution volumes of column eluants was very important in obtaining a good relation between the ratio of the elution volume, V/Vo, and the log of the molecular weight of proteins. Thus an accurately measured flow rate of the column was necessary. The flow rate of each column was measured after the column had been equilibrated with the borate-citrate buffer, pH 6.0, for at least 5 days after packing, and the flow rate was determined by collecting the column eluants in a 12-hour and 24-hour period for 2-3 days, and then converting to an hourly basis. A constant value expressed in ml/hr. was taken as the flow rate of the column. It is advisable to check the flow rate periodically to ensure a constant flow rate during the entire experiment.

Application of Sample to the Column. Correct application of the sample to the column was important in getting a good result. Before the Sample was applied, the effluent polyethylene tube was closed with a screw clamp and the eluant polyethylene tube was disconnected from the borate-

citrate buffer reservoir. The sample or the protein to be studied was dissolved in the equilibrating buffer (borate-citrate buffer, pH 6.0, 1 ml) and the solution applied as a layer on the nylon net of the sample applicator or Perspex. A syringe fitted with a long needle or a long polyethylene capillary tube was used to ensure even distribution of the sample on the gel surface. The sample should be more dense than the eluant. If the sample was not dense enough, sucrose may be added to increase the density of the sample. However, the proteins used in this investigation and component 1 were more dense than the borate-citrate buffer, pH 6.0.

Examination and Measurement of the Elution Volumes. Proteins were determined by a Recording Ultraviolet-Analyzer. An ISCO Recording Ultraviolet Analyzer, Model UA by Instrumentation Specialties Co., Inc., Lincoln, Nebraska, was used in this investigation.

Three methods were employed to determine elution volumes:

1. By means of the Recording Ultraviolet Analyzer (37). When the fraction of the column effluent containing the protein fraction passed through the optical unit of the Ultraviolet Analyzer, at 254 mμ, a peak would be recorded automatically on the chart in the Actuator. The speed of the chart was four inches to an hour. After the time taken for the development of the peak maxima was recorded and the flow rate of the eluent measured, the elution volume was calculated according to the formula:

$$\text{Elution Volume (ml)} = \text{Flow Rate (ml/hr)} \times \text{Time (hr)}$$

2. A more accurate way of measuring volume was achieved with a "Volumeter" ISCO Model V (47), in addition to the Recording Ultraviolet Analyzer. By means of the cooling effect of the column effluent on the warm thermistor, any definite amount of the column effluent was collected each time while the chart was marked with a dot for identification. By counting the dots

taken for the development of the peak maxima and measuring the volume collected in the Volumeter each time, the elution volume was calculated:

Elution Volume (ml) = Number of dots x Volume (ml)

3. At times when the Automatic Actuator failed to work, a manual determination was necessary. This was achieved by means of the "Fraction Collector" (37) attaching to the Optical Unit of the Ultraviolet Analyzer. The "Fraction Collector" allowed each test tube to collect a certain amount of effluent at a certain time interval. The contents of each test tube were tested with a Beckman Spectrophotometer in order to find the peak maxima. The elution volume was determined as described in paragraph 1.

EXPERIMENTAL AND RESULTS

In order to obtain a serum with significant amounts of component 1, cockerels were injected with diethylstilbestrol dissolved in propylene glycol (7.5 mg/0.5 ml/day). On the eighth day these birds were sacrificed and the serum obtained as described previously (page 6).

Ultracentrifugation was used as the initial step to separate the "Bottom Fraction" which contained a relatively high concentration of component 1, from the whole serum, as described on page 6.

After ultracentrifugation, the fractions were observed in the centrifuge tube and were designated as "Top Fraction", "Middle Fraction", and "Bottom Fraction" respectively according to their relative positions (Plate I). The "Top Fraction" appeared to be a yellowish waxy substance. The "Middle Fraction" was a yellow to orange color, thick, viscous liquid. The "Bottom Fraction" was a brownish red precipitate, and appeared to be waxy or semi-solid. In order to obtain the "Bottom Fraction", the "Top Fraction" was removed with a spatula and the "Middle Fraction" by decantation. The "Bottom Fraction" remaining in the centrifuge tube was dissolved in a minimum amount (1-2 ml) of the borate-chloride buffer, pH 8.6, and was subjected to a clarification by mild centrifugation at 5,000 r.p.m. for 30 minutes at 0° C. The supernatant was collected and used in the density-gradient centrifugation.

Density-gradient Centrifugation was used to separate component 1 from the other components of "Bottom Fraction". Previous studies (7) showed that after density-gradient centrifugation component 1 was concentrated in the precipitate.

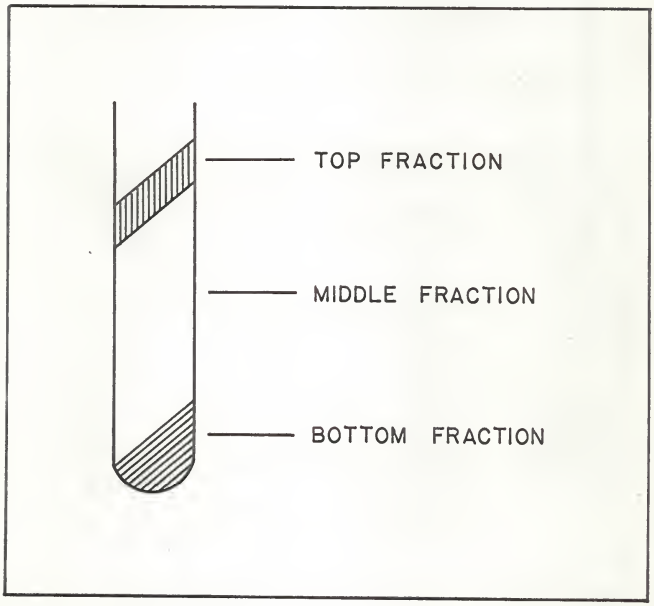
Sucrose solutions of 10%, 20%, 30%, 40%, and 50% in borate-chloride

EXPLANATION OF PLATE I

A schmatic diagram of diethylstilbestrol-treated cockerel serum fraction obtained upon centrifugation at 38,000 r.p.m. at 0° C. for 16 hours in a Spinco Ultracentrifuge Model L.

PLATE I

DIETHYLSTIBESTROL – INJECTED COCKEREL SERUM FRACTIONS



buffer, pH 8.6, were used as gradient-forming material. Lusteroid centrifuge tubes, 1" x 3", were filled with the gradient-forming agent. The sample, which was the "Bottom Fraction" in borate-chloride buffer, pH 8.6, was introduced at the top of the gradient, and centrifugation carried out as previously described (page 7).

In addition to several layers visible as bands in the centrifuge tubes, a yellowish, semi-solid substance was present at the bottom of the centrifuge tube after density-gradient centrifugation. The precipitate was obtained by pouring off the liquid layers. The precipitate was dissolved in a minimum amount (1-2 ml) of borate-citrate buffer, pH 6.0. Depending upon the amount available, 5-12 ml were dialyzed against borate-citrate buffer, pH 6.0, at 5° C. for 24 hours.

Characterization of Component 1. For the purpose of reference and characterization, a small amount of the component 1 prepared in the previous section was analyzed by means of vertical starch gel electrophoresis. The procedure for this electrophoresis was carried out following the method of Boyer (3) as described previously (page 9).

The starch gel was made from 45.45 gm of hydrolyzed starch suspending in 450 ml of gel buffer. The samples used were component 1, the sera of normal cockerels and the sera of diethylstilbestrol-treated cockerels. The electrophoresis was carried out at a current of 15 ma for 15 hours at room temperature.

The starch was dyed and washed, and after soaking in glycerine for at least a week, the results of the starch gel electrophoretic patterns were photographed. A representative illustration of these results isshown in Plate II. Patterns B and D were representative patterns of diethylstilbestrol-treated and normal cockerels, respectively. Patterns A and C were analyses

EXPLANATION OF PLATE II

Vertical starch gel electrophoretic patterns of :

A. Component 1 of the blood serum of diethylstilbestrol-treated cockerel.

B. Whole serum of diethylstilbestrol-treated cockerel.

C. Component 1 of the blood serum of diethylstilbestrol-treated cockerel.

D. Whole serum of normal cockerel.

The electrophoretic analyses were conducted under the conditions of:

Buffer	Gel buffer pH 8.6-8.7
	Wick and electrode chamber buffer, pH 8.0-8.2
Time	16 hours
Temperature	Room temperature
Current	15 ma

PLATE II

of the component 1 just prior to introduction into the Sephadex column. Both A and C showed one major component and two very minor components.

Thin-layer Chromatography was employed to detect the presence of lipids in component 1. The lipid (if any), of component 1 from the blood serum of the diethylstilbestrol-injected cockerel, was first extracted by a modification of the method of Selvey (39), and used as the sample in two-dimensional thin-layer chromatography described on page 11. Components were detected by iodine vapor in an enclosed tank, and were photographed. A representative illustration is shown on Plate III. Two minor components were visible in the developed chromatogram.

Gel Filtration. Sephadex G-100 (1.8 cm x 190 cm), G-100 (2.5 cm x 100 cm) and G-200 (2.5 cm x 100 cm) columns were employed. The preparation and packing of the Sephadex columns were carried out as described previously (page 13).

The inlet of the packed Sephadex column was connected by means of polyethylene tube to a reservoir of eluant (borate-citrate buffer, pH 6.0), and the outlet tube, fitted with a long polyethylene tube, passed through the cell of the Ultraviolet Analyzer which was connected to a recorder. The column effluent was collected in a beaker. In order to minimize the influence of temperature, the Sephadex column was kept at a constant temperature of 5° C.

The Determination of Void Volume. The proteins used for the determination of void volume must be completely excluded from the Sephadex gel. Gamma-globulin which was employed in the preliminary work gave a void volume (Vo) of approximately 138 ml after correction for the volume in the polyethylene tube on the Sephadex G-100 (1.8 cm x 190 cm) column. There were two components in gamma-globulin corresponding to two peaks in the Ultra-

EXPLANATION OF PLATE III

A representative of a thin-layer chromatogram of lipid extracted from component 1 of the blood serum of diethylstilbestrol-treated cockerels.

S. The position of the sample applied.

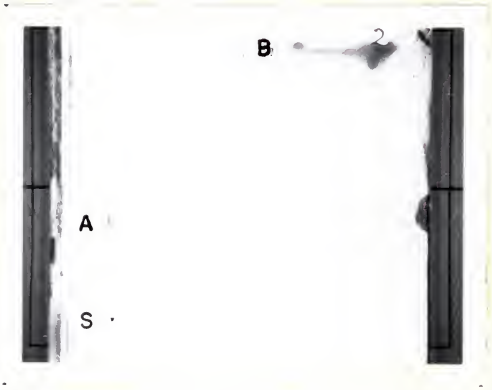
A. A minor component assumed to be cholesterol (44).

B. A minor component assumed to be phospholipid (lecithin) (44).

The two-dimensional chromatographic analysis was conducted under the conditions of:

First solvent	Chloroform : methanol : water (65 : 25 : 4)
Time	75 minutes
Second solvent	Heptane : ether : acetic acid (80 : 20 : 6)
Time	45 minutes
Temperature	Room temperature

PLATE III



violet Analyzing Recorder. The elution volume of the first peak was taken as the void volume of the column. The first peak was much sharper than the second peak. In later work, on both Sephadex G-100 and Sephadex G-200 column, 2.5 cm x 100 cm, Blue Dextran 2000, a blue Sephadex, was used to determine the void volume, Vo, of the column. The Blue Dextran 2000 gave only one peak on the Ultraviolet Analyzing Recorder. The peak was sharp and easily observable and therefore was considered more desirable than the gamma-globulin. Elution volume (corrected) of Blue Dextran 2000 on Sephadex G-100 was 155 ml, and on Sephadex G-200 was 116 ml.

Elution Volumes of Proteins of Known Molecular Weight. The proteins listed in Table I were used in preparing the standard curves for molecular weight determination. The proteins were applied to the sample applicator as previously described (page 15). Correct application of sample was important in order to obtain a good resolution.

Table 2 shows the results of elution volumes, V, of gamma-globulin, pepsin, alpha-chymotrypsinogen, trypsin, ribonuclease and cytochrome C on Sephadex G-100 column, 1.8 cm x 190 cm, using borate-citrate buffer, pH 6.0, at 5° C. as eluting agent. A gradual increase in elution volumes, V, was observed from gamma-globulin to cytochrome C.

Table 3 shows the results of elution volumes, V, of gamma-globulin, pepsin, alpha-chymotrypsinogen, and cytochrome C on Sephadex G-100 column 2.5 cm x 100 cm. Table 4 shows the results of elution volumes of proteins when Sephadex G-200 was employed. In both cases, the elution volumes, V, indicated a gradual increase from gamma-globulin, pepsin, alpha-chymotrypsinogen to cytochrome C.

The elution volumes, V, were determined from the peaks recorded on the Recorder of Ultraviolet Analyzer as previously described (page 16).

Table 1. Proteins used in gel filtration experiment.

Protein [1]	Description	Molecular Weight	Method [2]	Reference
Cytochrome C (from horse heart)	Amorphous powder	12,400	A.A.S.	Margoliash (27)
Ribonuclease (from ox pancreas)	5X crystalline	13,700	A.A.S.	Hirs et al (20)
Trypsin	2X crystalline	23,800	S.D.	Cunningham (11)
Alpha-chymotrypsinogen (from beef pancreas)	6X crystalline	25,000	Various [3]	Wilcox et al (49)
Pepsin	2X crystalline	35,500	S.D.	Yanari et al (51)
Gamma-globulin	Fraction II	160,000	U.	Phelps et al (34)

1. All proteins were obtained from Mann Laboratories, Inc., New York.

2. A.A.S., calculation (to the nearest 100) from the amino acid sequence;
S.D., sedimentation velocity diffusion; U., ultracentrifugal method.

3. Given in the reference cited as the best estimate from data by several authors.

Table 2. The summary of the ratio of elution volume of proteins used on Sephadex G-100 column, 1.8 cm x 190 cm.

Protein	Elution volume in ml.	Corrected volume in ml.	Ratio of elution volume,[1] V/Vo
Blue Dextran 2000		130[2]	
Gamma-globulin	164	138	1.06
Pepsin	262	234	1.815
Alpha-chymotrypsinogen	287	261	2.008
Trypsin	295	269	2.07
Ribonuclease	331	304	2.339
Cytochrome C	338	312	2.40

The equilibrating buffer was borate-citrate buffer, pH 6.0, at 5° C.

1. These are calculated values based on Blue Dextran 2000 as the void volume.

2. Calculated value based on the comparable result from the Sephadex G-100 column, 2.5cm x 100cm.

Table 3. The summary of the ratio of elution volume of proteins used on Sephadex G-100 column, 2.5 cm x 100 cm.

Protein	Elution volume in ml.	Corrected volume in ml.	Ratio of elution volume, V/Vo
Blue Dextran 2000	182	155	
Gamma-globulin (first peak)	192	162	1.013
Pepsin	305	278	1.794
Alpha-chymotrypsinogen	323	296	1.910
Cytochrome C	379	352	2.271

The equilibrating buffer was borate-citrate buffer, pH 6.0, at 5° C.

Table 4. The summary of the ratio of elution volume of proteins used on Sephadex G-200 column, 2.5 cm x 100 cm.

Protein	Elution volume in ml.	Corrected volume in ml.	Ratio of elution volume, V/Vo
Blue Dextran 2000	150	116	
Gamma-globulin (first peak)	203	169	1.457
Pepsin	290	256	2.207
Alpha-chymotrypsinogen	320	286	2.466
Cytochrome C	355	321	2.767

The equilibrating buffer was borate-citrate buffer, pH 6.0, at 5° C.

Calibration of Sephadex Columns for Molecular Weight Determination. Sephadex columns G-100, G-200 were calibrated with proteins of known molecular weight: gamma-globulin, pepsin, alpha-chymotrypsinogen, trypsin, ribonuclease and cytochrome C. Elution volumes, V, for these proteins were determined as described in the preceeding paragraph. The void volume, Vo, was also determined as described. Based on these, the ratio of elution volume, V, to the void volume, Vo, was calculated. Results of the ratio of elution volume for proteins on Sephadex G-100, 1.8 cm x 190 cm column are shown in Table 2, which were calculated values using Blue Dextran 2000 to determine the void volume. Results of the ratio of elution volume for proteins on Sephadex G-100 and G-200, 2.5 cm x 100 cm column, are shown in Table 3 and Table 4 respectively, both using the Blue Dextran 2000 to determine the void volume.

In each case, the ratio of elution volume to void volume, V/Vo, was plotted against the log of the molecular weight of proteins listed in Table 1. As shown in Fig. 1, Fig. 2 and Fig. 3 a linear relationship was observed between the ratio of elution volume, V/Vo, and the log of molecular weight for gamma-globulin, pepsin, alpha-chymotrypsinogen, trypsin, ribonuclease and cytochrome C.

Determination of Molecular Weight of Component 1. One ml of the yellow precipitate after density-gradient centrifugation, dissolved in a minimum amount of borate-citrate buffer, pH 6.0, and dialyzed against borate-citrate buffer, pH 6.0, for 24 hours at 5° C., was injected by means of a syringe as an evenly distributed layer on the nylon net of the sample applicator. The elution volume, V, of component 1 in Sephadex G-100 (1.8 cm x 190 cm), Sephadex G-100 (2.5 cm x 100 cm), and Sephadex G-200 (2.5 cm x 100 cm) columns was determined one after the other. The results are

shown in Table 5. The ratio of elution volume to void volume, V/Vo, for component 1 in Sephadex G-100 (1.8 cm x 190 cm) was 0.954, on Sephadex G-100 (2.5 cm x 100 cm) was 0.910 and Sephadex G-200 (2.5 cm x 100 cm) was 1.336, which corresponded to a log molecular weight of 5.25, 5.27, 5.28 respectively as shown in Fig. 1 Fig. 2 and Fig. 3.

Fig. 1. Plot of the ratio of elution volume, V/Vo, against the logarithm of molecular weight of proteins on Sephadex G-100, 1.8 cm x 190 cm column. The column was equilibrated with borate-citrate buffer, pH 6.0, at 5° C.

A. Cytochrome C

B. Ribonuclease

C. Trypsin

D. Alpha-chymotrypsinogen

E. Pepsin

F. Gamma-globulin

G. Component 1

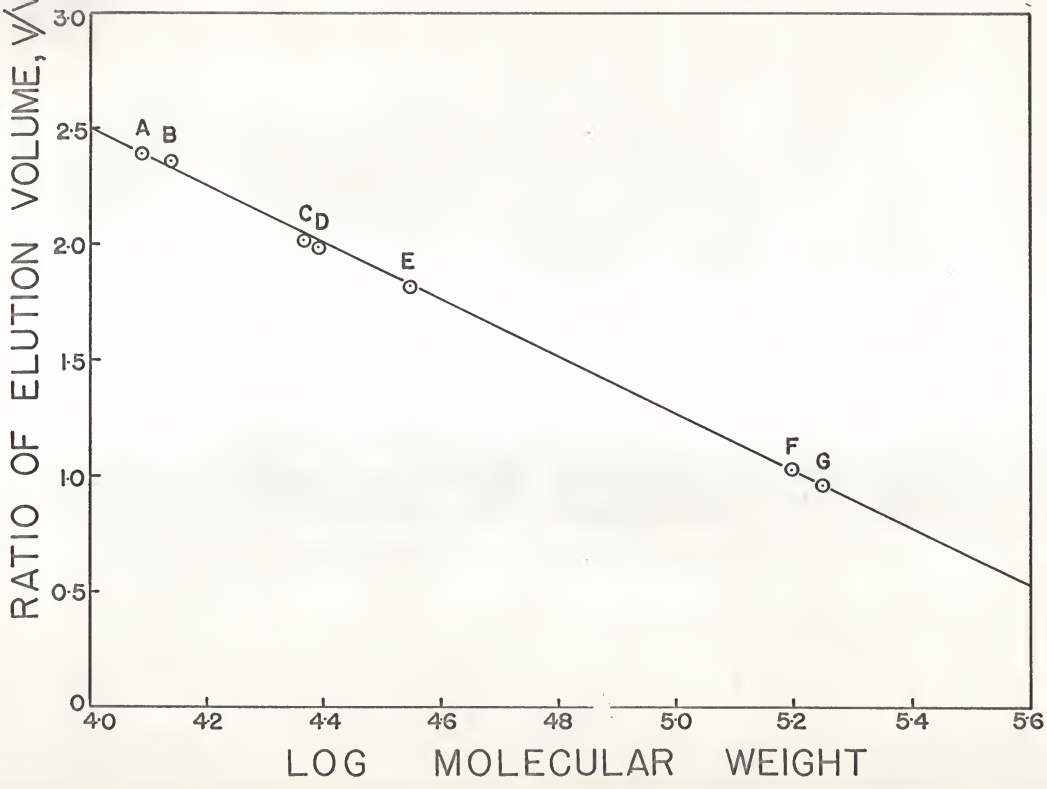

RATIO OF ELUTION VOLUME, V/V0
3·0
2·5
2·0
1·5
1·0
0·5
0
A
B
C
D
E
F
G
4·0
4·2
4·4
4·6
4·8
5·0
5·2
5·4
5·6
LOG MOLECULAR WEIGHT

Fig. 2. Plot of the ratio of elution volume, V/Vo, against the logarithm of molecular weight of proteins on Sephadex G-100, 2.5 cm x 100 cm column. The column was equilibrated with borate-citrate buffer, pH 6.0, at 5° C.

A. Cytochrome C

D. Alpha-chymotrypsinogen

E. Pepsin

F. Gamma-globulin

G. Component 1

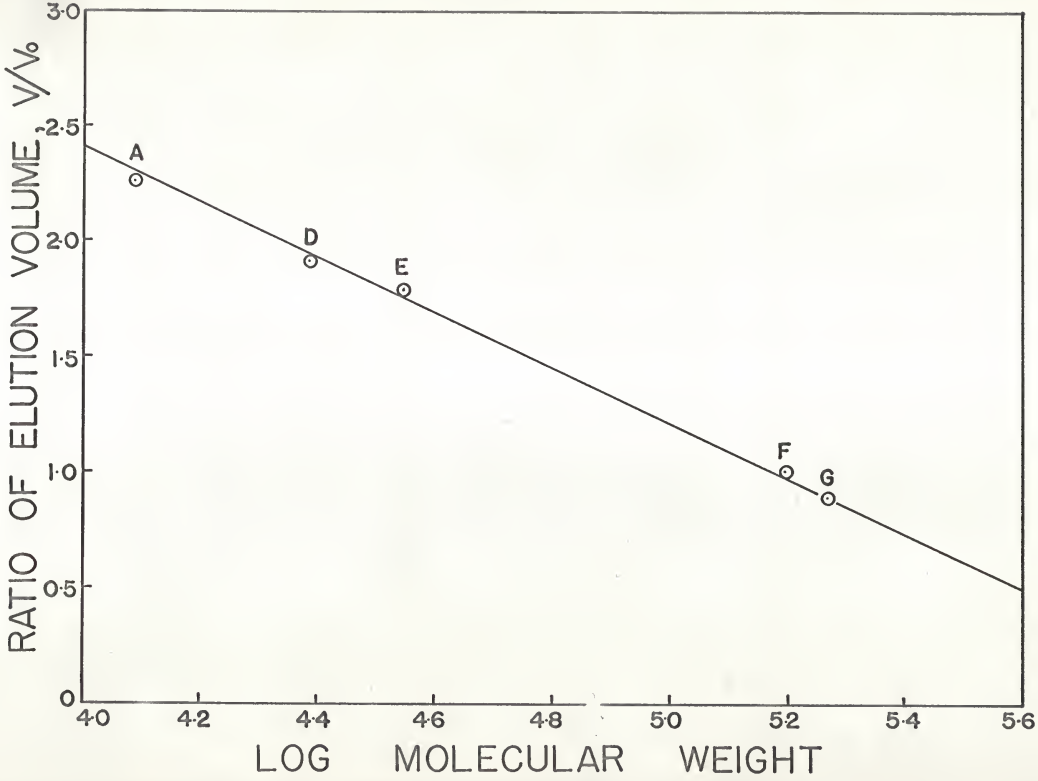

RATIO OF ELUTION VOLUME, V/V₀
3·0
2·5
2·0
1·5
1·0
0·5
0
A
D
E
F
G
4·0
4·2
4·4
4·6
4·8
5·0
5·2
5·4
5·6
LOG MOLECULAR WEIGHT

Fig. 3. Plot of the ratio of elution volume, V/Vo, against the logarithm of molecular weight of proteins on Sephadex G-200, 2.5 cm x 100 cm column. The column was equilibrated with borate-citrate buffer, pH 6.0, at 5° C.

A. Cytochrome C

D. Alpha-chymotrypsinogen

E. Pepsin

F. Gamma-globulin

G. Component 1

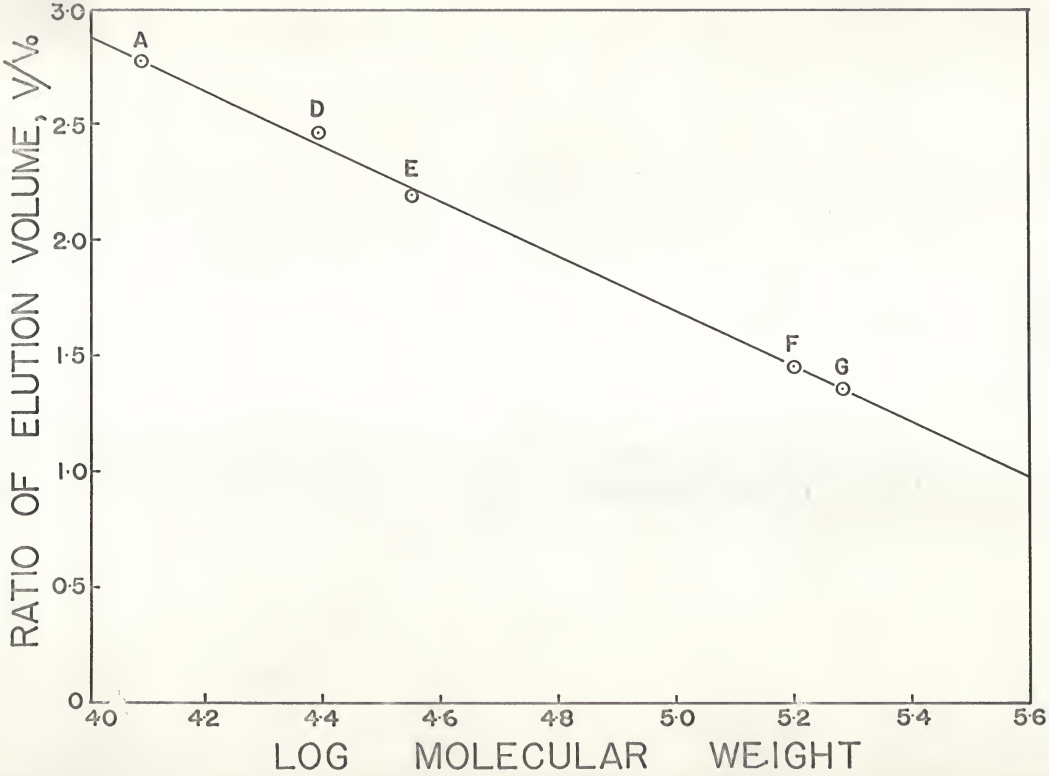

RATIO OF ELUTION VOLUME, V/V0
3·0
2·5
2·0
1·5
1·0
0·5
0
A
D
E
F
G
4·0
4·2
4·4
4·6
4·8
5·0
5·2
5·4
5·6
LOG MOLECULAR WEIGHT

Table 5. The summary of the elution volume, V, and the ratio of elution volume to void volume, V/Vo, of component 1 on different gel filtration columns.

Type of column	Elution volume in ml.	Corrected volume in ml.	Void volume in ml.	Ratio of elution volume, V/Vo
Sephadex G-100, 1.8 cm x 190 cm	150	124	130	0.954
Sephadex G-100, 2.5 cm x 100 cm	168	141	155	0.910
Sephadex G-200, 2.5 cm x 100 cm	189	155	116	1.336

The equilibrating agent used for all columns were borate-citrate buffer, pH 6.0, at 5° C.

DISCUSSION

Gel filtration has been used for the separation of mixtures, and, to a lesser extent, for the estimation of the molecular weights of polymers and proteins. Sephadex, cross-linked dextrans, are particularly good gel filtration media (25, 38, 18, 15, 48, etc.) In this investigation, Sephadex G-100 and Sephadex G-200 were employed as gel media to determine the molecular weight of component 1 of the blood serum of diethylstilbestrol-treated cockerels. Proteins with known molecular weights (Table 1) were used to test the correlation between molecular weight and gel filtration behavior, and to establish calibration curves. Two different columns (1.8 cm x 190 cm and 2.5 cm x 100 cm) were employed.

The amount of proteins employed depended on the sensitivity of the instrument used to detect the concentration of the column effluents. Previous work has shown that concentration and size of samples would not affect the ratio of elution volume, V/Vo (48). In this experiment, with the less sensitive range (0-2.5) of the Recording Ultraviolet Analyzer, 7-17 mg of the purified proteins (Table 1) dissolved in 1 ml of the equilibrating buffer, borate-citrate buffer, pH 6.0, were sufficient to obtain well defined peaks on the chart of the recorder.

The ratio of the elution volume to void volume, V/Vo, for cytochrome C, pepsin, alpha-chymotrypsinogen and gamma-globulin were found to be relatively constant for the two columns used when Sephadex G-100 was employed as gel media (Table 2 and Table 3). With Sephadex G-100 and Sephadex G-200 on the 2.5 cm x 100 cm column, although the ratio of elution volume, V/Vo, was found to be different, the results (molecular weight determination) of the different Sephadex gels

(Sephadex G-100 and Sephadex G-200) were comparable (Fig. 2 and Fig. 3).

A linear relationship between the ratio of elution volume, V/Vo, and the log of molecular weight of proteins was obtained for Sephadex G-100 on 1.8 cm x 190 cm column, and Sephadex G-100 and Sephadex G-200 on 2.5 cm x 100 cm (Fig. 1, Fig. 2 and Fig. 3). This agreed with the results obtained by Whitaker (48) with these proteins on Sephadex G-75 and Sephadex G-100. The ratio of elution volume, V/Vo, for component 1 as determined on Sephadex G-100, 1.8 cm x 190 cm column was 0.954, on Sephadex G-100, 2.5 cm x 100 cm column was 0.910, and on Sephadex G-200, 2.5 cm x 100 cm was 1.336 (Table 5). Using the calibration curves obtained from these columns with their respective gel media, and the ratio of elution volume, V/Vo, of component 1, the molecular weight of component 1 was determined. The calculation gave molecular weights of 1.78×10^5, 1.8×10^5, and 1.9×10^5 respectively for component 1.

The most likely cause of error in the determination of protein molecular weight by gel filtration is the inaccurate measuring of elution volumes. The density differences between solvated protein molecules would also result in a different ratio between size and molecular weight. Moreover, the protein molecules may be affected by an interaction with the acidic groups of the Sephadex gels (16, 14) causing retention of strongly basic proteins on the gels (10), and exclusion of strongly acidic macromolecules from the inner cavities (19, 35). However, moderate salt concentration appeared sufficient to overcome the retention of proteins on Sephadex gels (17).

In the preliminary work on the long column (1.8 cm x 190 cm) the molecular weight of component 1 was shown to be greater than the molecular

weight of gamma-globulin. Therefore it was desirable to use a substance with molecular weight higher than the proteins used (Table 1) and component 1 to determine the void volume. This led to the use of Blue Dextran 2000 to determine the void volume and the use of Sephadex G-200 in the later experiments on the shorter column (2.5 cm x 100 cm).

The Sephadex G-200 appeared to give sharper elution curves and a better correlation between the elution volume, V/Vo, and molecular weight. It should be noted that Sephadex G-100 is more highly recommended than Sephadex G-75 for determining molecular weight up to approximately 1.5×10^5 (1). Since the molecular weight of component 1 is greater than 1.5×10^5 the next higher Sephadex (G-200) was probably better for this work. However, as long as the elution rate was slow, both Sephadex G-100 and Sephadex G-200 appeared to give good results.

Wu (50) showed, using moving boundary electrophoretic studies, that the electrophoretic pattern of the precipitate obtained from density-gradient centrifugation indicated only one peak which could be identified as the prealbumin of the original blood serum of diethylstilbestrol-treated cockerels. In the present investigation as a result of vertical starch gel electrophoresis, the electrophoretic pattern of the precipitate obtained from density-gradient centrifugation showed one major component and two minor components. Moreover, component 1 did not migrate in front of the albumin as in the case of free electrophoresis. The retardation of migration of component 1 indicated that the molecular weight of component 1 was greater than that of albumin--the molecular weight of serum albumin is 70,000 (23)--because the starch gel has a gel filtration effect which is more apparent on higher molecular weight proteins. The fraction which was analyzed on Sephadex column was the major component

since the minor components were too dilute to appear on the Ultraviolet Recordor.

The lipid extracted from component 1 was subjected to two-dimensional thin-layer chromatography. The chromatographic pictures showed only two minor components which were probably cholesterol (A of Plate III) and Phospholipid (lecithin) (B of Plate III) (44). The small amount present indicated that component 1 is probably not a lipoprotein and that these traces of lipids may be impurities. This result agreed with Wu (50). With sodium dextran sulfate treatment, Wu found that the size of component 1 in the electrophoretic pattern was not affected by the dextran sulfate, which indicated that component 1 did not form a complex with anionic polysaccharides, substances that appear to be specific for precipitating lipoproteins.

SUMMARY

1. Component 1, which is present in the blood serum of diethylstilbestrol-treated cockerels, was separated by ultracentrifugation and purified by density-gradient centrifugation.

2. The homogeneity was tested by vertical starch gel electrophoresis and was shown to be composed of one major component and two very minor components.

3. Thin-layer chromatography was employed to determine the amount of lipid present. The results indicated that only a trace of lipid was present and this may be present as an impurity.

4. The molecular weight of component 1 as determined by the gel filtration technique was $1.83 \pm 0.05 \times 10^5$.

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MOLECULAR WEIGHT DETERMINATION OF COMPONENT I OF THE BLOOD SERUM OF DIETHYLSTILBESTROL-TREATED COCKERELS

by

JANE C. S. SHAW

A.B., University of California, Berkeley, 1963

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

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Component 1, a prealbumin found in the electrophoretic pattern of the blood serum of diethylstilbestrol-treated cockerels, has been found to be concentrated in the "Bottom Fraction" after ultracentrifugation. By means of density-gradient centrifugation, a purified component 1 was isolated as a precipitate from the "Bottom Fraction".

Vertical starch gel electrophoresis was employed to compare the electrophoretic patterns of component 1, the serum of diethylstilbestrol-treated cockerel, and the serum of normal cockerel. The electrophoretic pattern of component 1 showed one major component and two very minor components. Component 1 did not move in front of the albumin as in the case of moving boundary electrophoresis, but was retarded considerably in its migration. This indicated that the molecular weight of component 1 was greater than that of albumin.

The lipid extracted from component 1 was used as the sample in the two-dimensional thin-layer chromatography. The chromatographic separation revealed two very minor components which when compared with the results of other investigators were assumed to be cholesterol and phospholipid (lecithin). The small amount present together with the results of Wu indicated that they probably were impurities.

The molecular weight of component 1 was determined by the gel filtration technique employing Sephadex gel media. Purified proteins of known molecular weights were used to calibrate the standard curves. The elution volumes, V, of these purified proteins were determined, and the void volume, Vo, was determined with Blue Dextran 2000. Then the ratio of elution volume to the void volume, V/Vo, was calculated. Graphs of V/Vo against the log of molecular weight of proteins employed were plotted for Sephadex G-100 (1.8 cm x 190 cm column), Sephadex G-100 (2.5 cm x 100

cm column), and Sephadex G-200 (2.5 cm x 100 cm column). A linear relationship between the ratio V/Vo and the log of molecular weight of proteins employed was observed in all three cases.

The elution volume, V, and subsequently the ratio V/Vo of the purified component 1 obtained from density-gradient centrifugation was determined on the Sephadex columns. By means of the three calibration curves previously obtained on the corresponding Sephadex column, the log of molecular weight of component 1 was read directly from the respective graphs. The molecular weight of component 1 of the blood serum of diethylstilbestrol-treated cockerels on Sephadex G-100 (1.8 cm x 190 cm column) was 1.78×10^5, on Sephadex G-100 (2.5 cm x 100 cm column) was 1.8×10^5, and on Sephadex G-200 (2.5 cm x 100 cm column) was 1.9×10^5 as determined by this gel filtration technique. The average was $1.83 \pm 0.05 \times 10^5$.